

Background

- During HAART the number of HIV-1 infected cells *in vivo* rapidly declines due to the short life span of activated/infected T-lymphocytes, killing by cytotoxic T-cells, and the intrinsic cytotoxicity of HIV-1. Nonetheless, even after several years of successful HAART, substantial numbers of infected memory T-lymphocytes still remain presumably expressing very low levels of viral proteins (latent reservoir). A new class of anti-HIV compounds that selectively kill HIV-1 infected cells may reduce the size of the latent reservoir when used in combination with HAART.
- Since HIV-1 infection affects normal cellular physiology, infected cells could be more vulnerable than uninfected cells to inhibitors of specific cellular enzymes. In support of this premise, several classes of compounds including cyclin dependent kinase (CDK) inhibitors have been identified¹ that selectively kill HIV-1 infected cells.

¹ Wang D, De la Fuente C, Deng L, et al. Inhibition of human immunodeficiency virus type 1 transcription by chemical cyclin-dependent kinase inhibitors. *J. Virol.* Aug 2001;75(16):7266-79.

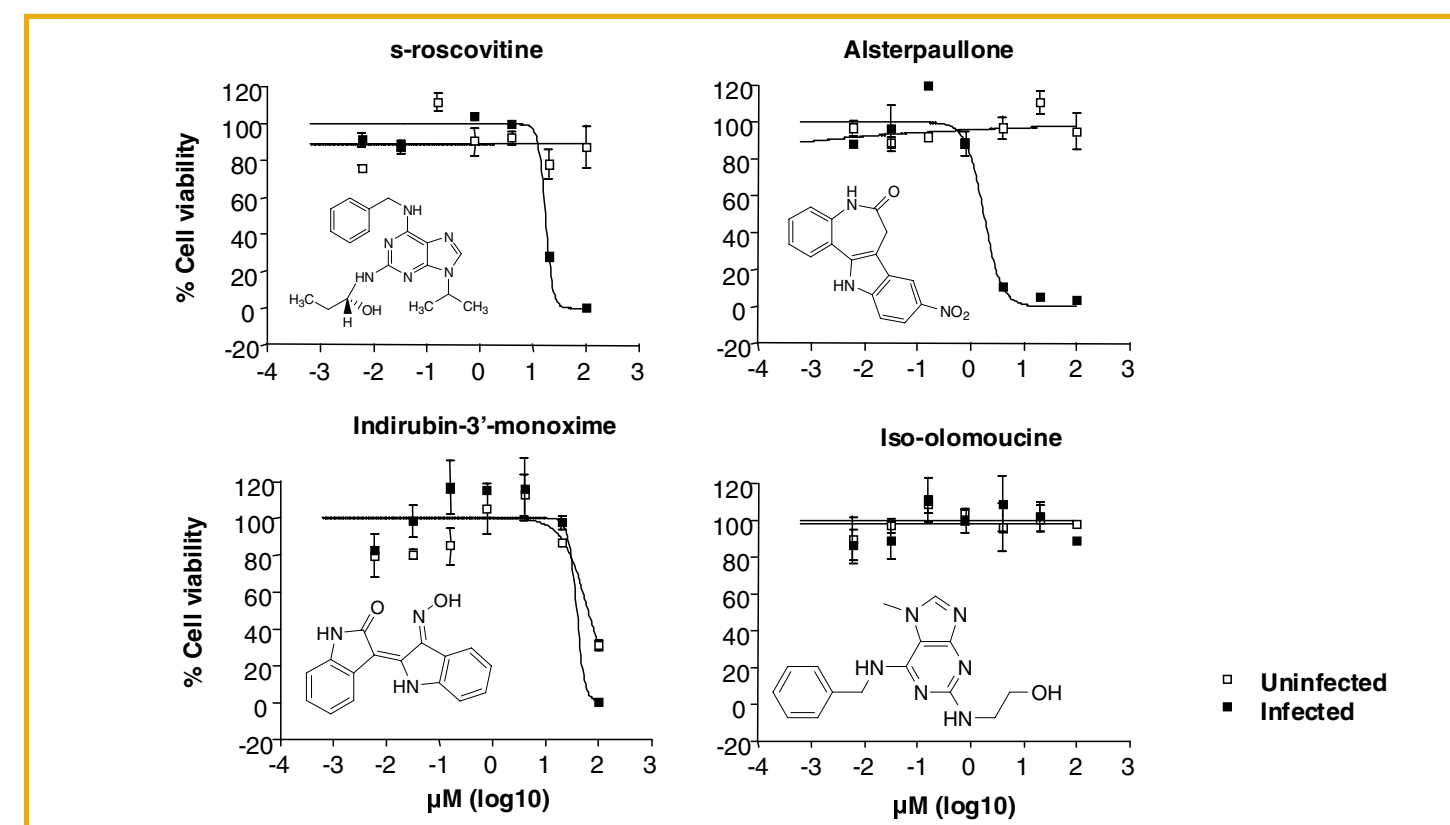
Objectives

- Set up a primary screening assay for selective killing of HIV-1 infected cells.
- Test CDK inhibitors for HIV-1 infected specific cell death.
- Test most active inhibitors for anti-HIV activity in primary T-lymphocytes and macrophages.

Methods

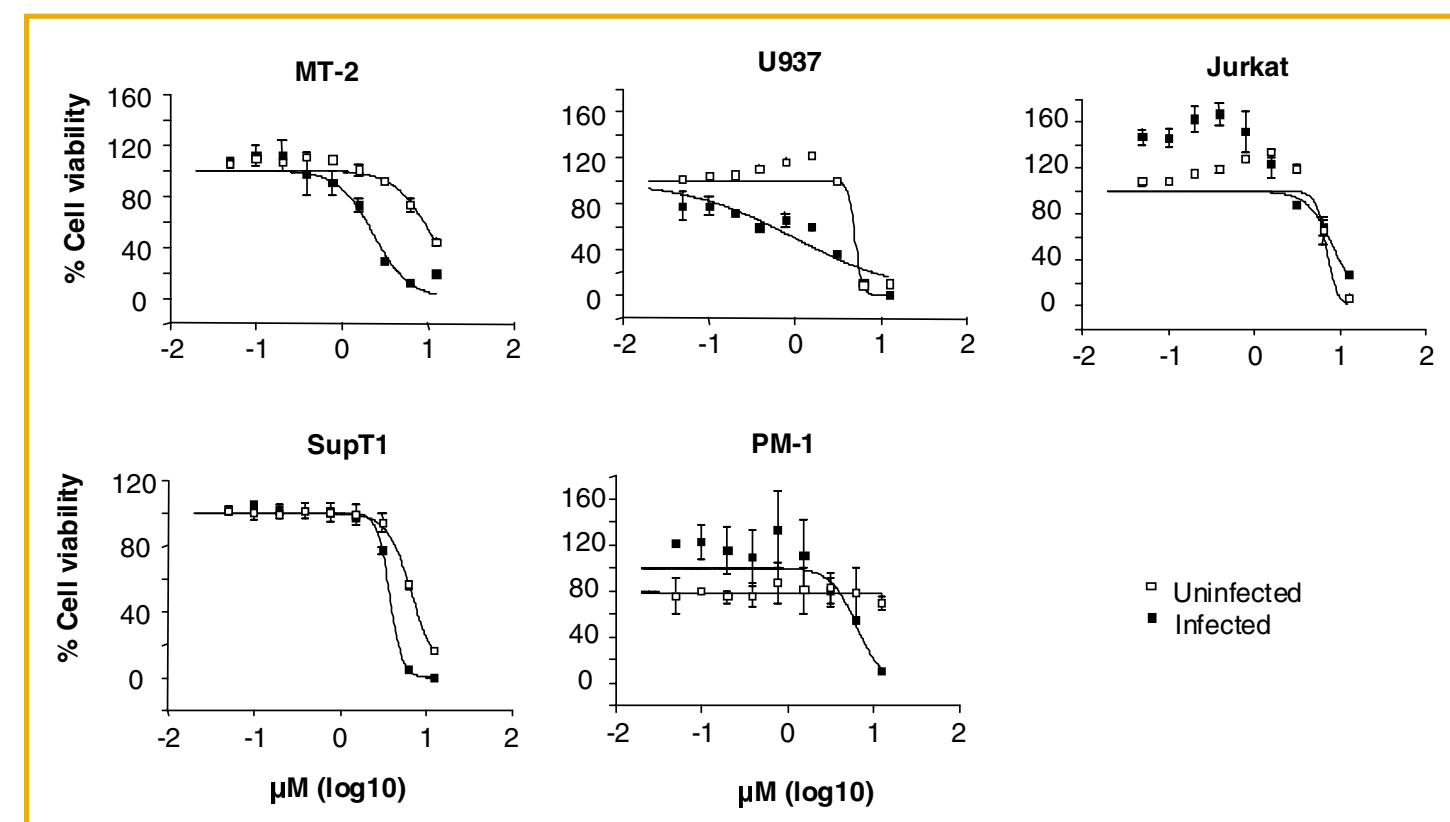
- Cell culture:** Human lymphoid cell lines, CEM, PM-1, SupT1, MT-2, Jurkat, and the monocytic cell line U937 were maintained in RPMI1640 medium supplemented with 10% FBS, 2mM L-glutamine and antibiotics (Complete Media). Monocytes were purified from peripheral blood mononuclear cells (PBMCs) using CD14-magnetic beads and differentiated to macrophages in Complete Media supplemented with 25ng/ml MCSF. Primary T-lymphocyte cultures were initiated by stimulating CD14-depleted PBMC with 1 µg/ml of phytohemagglutinin (PHA) and IL-2 in Complete Media supplemented with 20% FBS.
- Chronically infected cells:** ACH-2 cells are CEM-derived cells chronically infected with HIV-1_{LAI}. To generate other chronically HIV-1 infected cells various cell lines were infected with HIV-1_{IIB} at a multiplicity of infection (MOI) equal to 0.001. Cells were maintained for at least six months of continuous passaging. By this time all cells are infected.
- Cytotoxicity assay for uninfected and chronically infected cell lines:** Chronically infected and uninfected cell lines were plated in 384-well culture plates and incubated with compounds for 20 hours. Cell viability was measured by Celltiter-Glo cell viability assay. Alternatively, cells were plated in 96-well plates incubated with compounds for 20 hours and cell viability was measured by XTT assay.
- Antiviral assay in primary T-cells and macrophages:** Primary T-lymphocytes and macrophages were infected with HIV-1_{Bal} strain at approximately 0.0001 MOI and 0.01 MOI respectively for 3 hours then treated with serially diluted compounds and cultured for four or five days. HIV-1 production was measured in culture supernatants by p24 antigen ELISA assay.
- Flow cytometry:** PHA-stimulated PBMCs were infected with HIV-1_{Bal} and cultured for 4-7 days. Drug treatment was initiated when 30-50% of activated lymphocytes became p24 positive. Twenty hours after addition of compounds, cell suspensions were stained for intracellular p24 and analyzed by flow cytometry.

Figure 1. Differential Susceptibility of Infected and Uninfected CEM Cells to CDK Inhibitors



- Uninfected CEM cells (open squares) and chronically infected CEM cells (closed squares) were treated with serial 5-fold dilutions of compounds. While s-Roscovitine and Alsterpaullone showed selective toxicity in infected cells, Indirubin-3'-monoxime was equally toxic in both infected and uninfected cells. Iso-Olomoucine was not toxic in either cells at the highest concentration tested (100µM).

Figure 2. HIV-1 Specific Cytotoxicity of Alsterpaullone in Other Cell Lines



- Pairs of uninfected (open squares) and chronically infected cells (closed squares) of MT-2, U937, Sup T1, PM-1, and Jurkat cell lines were treated with serial 2-fold dilutions of Alsterpaullone for 20 hours. Selectivity was observed in MT-2, U937, and PM-1 cells but not in Jurkat and Sup T1 cells.

Results

Table 1. Screening of Various CDK Inhibitors and Related Molecules for HIV-1 Specific CEM Cell Killing

Selectivity	Name	CC ₅₀ in µM		Selectivity ¹	Reported activities to kinases (IC ₅₀ in nM) ²
		Infected	Uninfected		
High (>10 fold)	Alsterpaullone	1.8	>200	>109	CDK1(35), CDK2(15), CDK5(40)
	CDK Inhibitor, p35	7	>200	>29	CDK1(100), CDK2(80)
	Kenpaullone	16.4	>200	>12	CDK1(400), CDK2(680), CDK5(850)
	s-Roscovitine	16.7	>200	>12	CDK1(650), CDK2(700), CDK5(160), CDK7(500)
Moderate (5-10 fold)	Aloisine A	19	>200	>11	CDK1(150), CDK2(120), CDK5(200)
	Bohemine	20.9	>200	>9.6	CDK1(1000)
	r-Roscovitine	23.7	>200	>8.5	CDK1(650), CDK2(700), CDK5(160), CDK7(500)
	Purvalanol-A	16.6	>122	>7.3	CDK1(4), CDK2(70), CDK5(75)
	CGP 74514-A	6.4	37.2	6	CDK1(25)
Poor (< 5 fold)	Indirubine-3'-monoxime,5-Iodo	11.9	53.9	4.5	CDK1(25), CDK5(20)
	Olomoucine	47.2	>200	>4.2	CDK1,2 (7000), CDK5(3000)
Unknown	Olomoucine, N9-Isopropyl	50.7	>200	>4	CDK1(2000)
	Compound 52	69.8	>153	>2.2	CDK1(340)
	Indirubin-3'-monoxime	21.6	46	2	CDK1(180), CDK2(250), CDK4(3330), CDK5(100)
	2,6-dichloropurine	105.6	>200	>1.9	
	2,6-Diaminopurine	28.3	45.6	1.61	
	WHI-P180	109.3	>158	>1.44	CDK2(1000)
	Flavone	>180	178.5	<1	CDK1(300), CDK2(100), CDK4(400), CDK7(300)
	6-BenzoyloxyPurine	>200	106.5	<0.5	
	9-Cyanopaullone	>102	>186	n/a	CDK1(24), CDK5(44)
	Compound 3	>141	>184	n/a	CDK2(60)
	Indirubin-3'-monoxime-5-sulphonic acid	>161	>184	n/a	CDK1(5), CDK5(7)
	CDK2/Cyclin Inhibitory Peptide 1	>167	>183	n/a	
	Butyrolactone I	>18	>18	n/a	CDK1(680)
	CDK2 Inhibitor peptide	>181	>119	n/a	CDK2(38)
	CDK2/5 Inhibitor	>200	>200	n/a	CDK2,5(2000)
Dimethylamino-Olomoucine	>200	>200	n/a	CDK1(>500,000), CDK4,5(>1,000,000)	
N-6(Δ2-isopentyl)-Adenine	>200	>200	n/a	CDK1,2,5(>50,000)	
Iso-Olomoucine	>200	>176	n/a	CDK1(>500,000), CDK4,5(>1,000,000)	
6-DimethylaminoPurine	>200	>164	n/a	cdc	

¹ CC₅₀ (50% cytotoxic concentration) was determined 20 hours after treatment with compounds. The average of 2-6 experiments is shown.

² Selectivity was calculated by dividing CC₅₀ in uninfected cells by CC₅₀ in infected cells.

³ IC₅₀ reported in the manufacturer's product data sheet, unless indicated otherwise.

Table 2. Combining Two Poorly Selective CDK Inhibitors Gives Better Selectivity in Chronically-infected CEM Cells

Selectivity	Compound A	Compounds used in combination*	CC ₅₀ in µM ¹		Selectivity ²
			Infected	Uninfected	
High (>10 fold)	Indirubine-3'-monoxime,5-Iodo	9-Cyanopaullone	7.1	>342	>48
	Indirubin-3'-monoxime	9-Cyanopaullone	10.6	>296	>28
	9-Cyanopaullone	Compound 3	22.6	>400	>18
	Indirubin-3'-monoxime-5-sulphonic acid	Indirubin-3'-monoxime,5-Iodo	27.4	>328	>12
Moderate (5-10 fold)	Indirubin-3'-monoxime	Indirubin-3'-monoxime-5-sulphonic acid	33.8	>296	>8.8
	Indirubin-3'-monoxime	CDK2 Inhibitor peptide	27.4	>236	>8.6
	Indirubin-3'-monoxime	Indirubine-3'-monoxime,5-Iodo	9.9	68.8	7.0
	Indirubine-3'-monoxime,5-Iodo	CDK2/Cyclin Inhibitory Peptide 1	27.4	187	6.8
	Indirubin-3'-monoxime-5-sulphonic acid	9-Cyanopaullone	47	>306	>6.5
Poor (<5 fold)	Indirubine-3'-monoxime,5-Iodo	None	11.9	53.9	4.5
	Indirubin-3'-monoxime-5-sulphonic acid	Compound 3	68.2	>272	>4
	Indirubin-3'-monoxime	Compound 3	21.8	87	4.0
	Indirubin-3'-monoxime	CDK2/Cyclin Inhibitory Peptide 1	34.6	100	2.9
	9-Cyanopaullone	CDK2/Cyclin Inhibitory Peptide 1	160.2	>400	>2.5
	Compound 3	CDK2/Cyclin Inhibitory Peptide 1	166.8	>400	>2.4
	Indirubin-3'-monoxime-5-sulphonic acid	CDK2 Inhibitor peptide	118.8	290	>2.4
	9-Cyanopaullone	CDK2 Inhibitor peptide	141.6	>330	>2.3
	Indirubin-3'-monoxime-5-sulphonic acid	CDK2 Inhibitor peptide	170.6	>334	>2
	Indirubin-3'-monoxime	None	21.6	46.0	2
Unknown	Indirubine-3'-monoxime,5-Iodo	CDK2 Inhibitor peptide	57.3	109.5	1.9
	Compound 3	CDK2 Inhibitor peptide	151.6	195.6	1.3
	CDK2 Inhibitor peptide	None	>181	>119	n/a
	CDK2/Cyclin Inhibitory Peptide 1	None	>167	>183	n/a
	Indirubin-3'-monoxime-5-sulphonic acid	None	>161	>184	n/a
Unknown	CDK2/Cyclin Inhibitory Peptide 1	None	>306	>364	n/a
	Compound 3	None	>141	>184	n/a
	9-Cyanopaullone	None	>102	>186	n/a
	Indirubin-3'-monoxime	None	>102	>186	n/a

* Seven CDK inhibitors (highlighted) that are either poorly selective or poorly toxic were used in two-drug combination at 1:1 molar ratio.

¹ CC₅₀ (50% cytotoxic concentration) was determined 20 hours after treatment with compounds. Average of 2-6 experiments are shown. When two compounds were tested in combination, the concentration represents the total of the two drugs.

² Selectivity was calculated by dividing CC₅₀ in uninfected cells by CC₅₀ in infected cells.

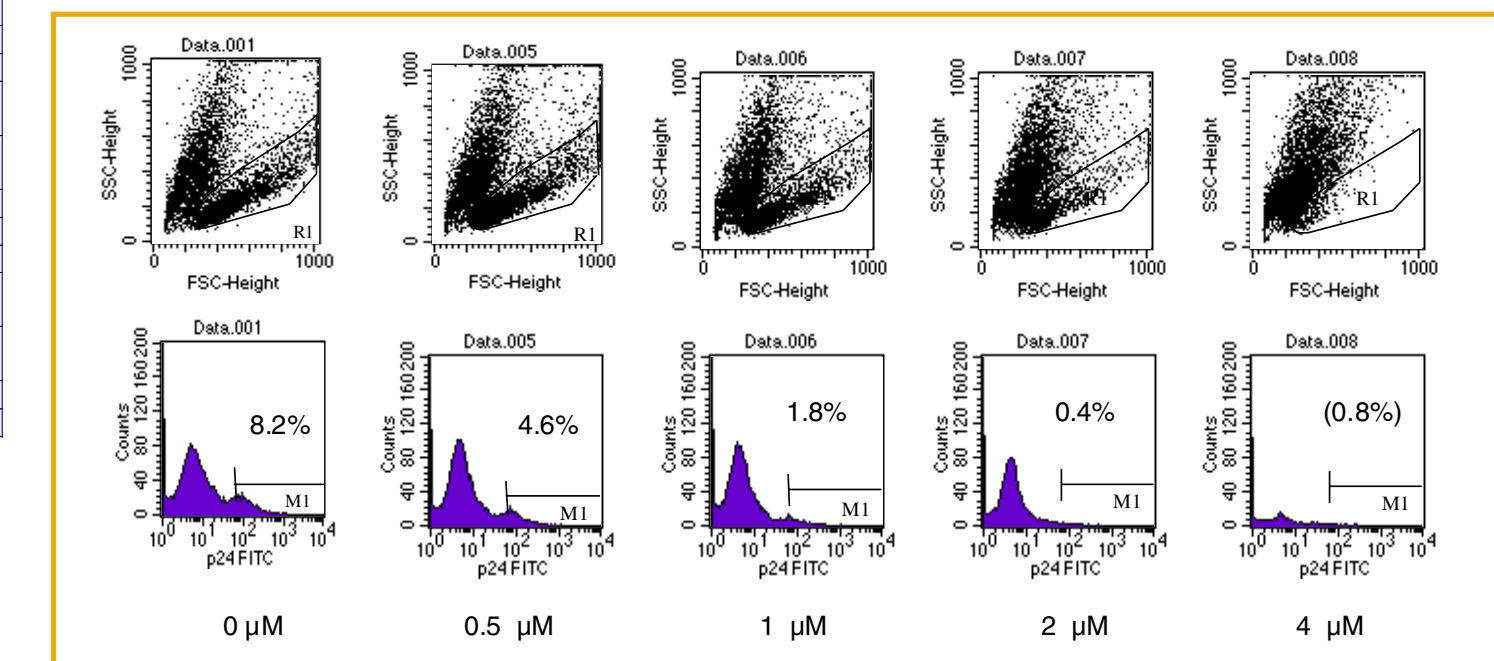
Table 3. Antiviral Activity of CDK Inhibitors in Primary T-Lymphocytes and Macrophages

Compound	Macrophages		T-lymphocytes	
	EC ₅₀ (µM)	CC ₅₀ (µM) ¹	EC ₅₀ (µM)	CC ₅₀ (µM) ¹
r-Roscovitine	.712 ± .314	>5	>3.131 ± 1.134	>5
s-Roscovitine	.676 ± .310	>5	>3.035 ± 1.103	>5
Purvalanol-A	2.314 ± 1.174	>5	TBD	>5
Olomoucine	2.816 ± .915	>5	>5	>5
Indirubin-3'-monoxime	.623 ± .476	>5	>5	>5
Indirubin-3'-monoxime-5-sulphonic acid	4.865 ± .207	>5	>5	>5
Indirubin-3'-monoxime-5-Iodo	.753 ± .804	>5	1.938 ± .739	>5
Kenpaullone	1.265 ± .550	>5	3.324 ± .746	>5
Alsterpaullone	1.234 ± .202	>5	.292 ± .197	>5
Butyrolactone 1	>5	>5	>5	>5
CGP 74514-A	2.007	>5	2.984	>5

¹ EC₅₀ (50% inhibitory concentration) was calculated from p24 production, 4 days after infection /compound treatment. Average (± SEM) of 2-10 experiments are shown.

² CC₅₀ (50% cytotoxic concentration) was calculated from % survival (measured by XTT assay) of uninfected cells, 4 days after compound treatment.

Figure 4. Dose-Response Decline of p24 Positive Cells in HIV-1 Infected PBMCs Cultures Treated with Alsterpaullone



- Infected PBMCs were treated with different concentrations of alsterpaullone for 20 hours, stained with anti-p24 antibody, and analyzed by flow cytometry. Top: Forward/side scatter cytogram. R1 represents the population of activated lymphocytes. Bottom: p24 expression in activated lymphocytes. Percent p24 positive cells are shown in the histograms.

Conclusions

- Using T-cell lines uninfected and chronically infected with HIV-1 we were able to screen various CDK inhibitors for selective killing of HIV-1 infected cells.
- This has led to the discovery of several classes of CDK inhibitors that also have antiviral activity in primary T-lymphocytes and macrophages.
- Although selectivity may be a limitation for this class of inhibitors, combinations of inhibitors may provide greater selectivity.